

10/031530
JC10 Rec'd PCT/PTO 17 JAN 2002

TRANSMITTAL FOR ENTRY OF PCT APPLICATION IN U.S. NATIONAL STAGE UNDER 35 U.S.C. § 371	Docket No.: P-UX 5156
Proposed Preliminary Classification: (optional)	Class: Subclass:

Address to: COMMISSIONER FOR PATENTS
Box PCT
Attn: US/EO
Washington, D.C. 20231



CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"EXPRESS MAIL" MAILING LABEL NUMBER: EL 857 043 455 US

DATE OF DEPOSIT: 17 January 2002

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING
DEPOSITED WITH THE UNITED STATES POSTAL SERVICE
"EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER
37 C.F.R. 1.10 ON THE DATE INDICATED ABOVE, AND IS
ADDRESSED TO: BOX PCT, COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231, ATTENTION: US/EO.

Craig Worthem
(TYPED OR PRINTED NAME OR PERSON MAILING PAPER OR FEE)

(SIGNATURE OF PERSON MAILING PAPER OR FEE)

This is a request for International Application **PCT/EP99/05234**
to enter U.S. national stage under 35 U.S.C. § 371. Commencement
of national stage processing of this application is expressly
requested under § 371(f).

International filing date: **22 July 1999**

Earliest priority date claimed: **22 July 1999**

Title: **METHOD FOR THE SPECIES-SPECIFIC DETECTION OF
ORGANISMS**

Inventors: **KRUPP, Guido; SCHEINERT, Peter; SÖLLER, Rainer;
SPENGLER, Ulrich**

Items required upon filing:

☒ Return receipt postcard

☐ Copy of the PCT application

☐ pages of the PCT application enclosed.

A copy of the PCT application does not need to be filed if a
pink Form PCT/IB/308 has been received in the PCT
application and it gives notice that the International
Bureau has communicated a copy of the application the U.S.
designated office.

☒ Form PCT/IB/308 was mailed on **01 February 2001**

☒ A check in the amount of **\$1,548** is enclosed to cover the
Basic National fee and any Excess Claim fees, calculated as
follows (payment of the fee cannot be deferred until after
filing)

☐ Small Entity Status is hereby asserted.

Inventors: **Krupp et al.**
Docket No.: **P-UX 5156**
Page 2

Basic National fee under § 1.492(a):

Ch. I search fee was paid to _____ USPTO **X** EPO

Ch. II int'l preliminary examination
fee (IPE fee) was paid to _____ USPTO **X** EPO

	Small entity*	Other entity*
Ch. II IPE fee was paid to the USPTO	___ \$ 355	___ \$ 710
No Ch. II IPE fee was paid to the USPTO, but Ch. I search fee was paid to USPTO	___ \$ 370	___ \$ 740
Neither the IPE fee nor the search fee was paid to the USPTO	___ \$ 520	___ \$1040
Ch. II IPE fee was paid to the USPTO, and the IPER states that all claims meet requirements of novelty, inventive step and industrial applicability [see IPER - if "yes" on all, and no "no", then choose this]	___ \$ 50	___ \$ 100
Ch. I search fee was paid to EPO or JPO [overrides "Neither the IPE fee nor the search fee was paid to the USPTO"]	___ \$ 445	<u>X</u> \$ 890

*new fees as of October 1, 2001

Excess claim fees under § 1.492(b), (c), (d):

	Number Filed		Number Extra		Rate			Fee	
					Small* Entity	Other* Entity		Small Entity	Other Entity
Total Claims	41-20	=	21	x	\$9	\$18	=	\$	\$378
Indepen- dent Claims	2 - 3	=	0	x	\$42	\$84	=	\$	\$0
Multiple Dependent Claims Presented: <u>X</u> Yes ___ No					\$140	\$280		\$	\$280
EXCESS CLAIM FEE								\$	\$658

*new fees as of October 1, 2001

X The Commissioner is hereby authorized to charge any fees required under 37 C.F.R. § 1.492 or § 1.17 or to credit any overpayment to Deposit Account No. 03-0370. A duplicate copy of this transmittal is enclosed for this purpose.

10/031530
531 Rec'd PCT/PT 17 JAN 2002

Inventors: Krupp et al.
Docket No.: P-UX 5156
Page 3

Items that are optional or may be deferred:

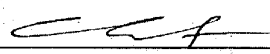
___ Pages of an executed Declaration for Patent Application
___ An executed Power of Attorney for Patent Application by
___ Assignee
___ Executed Statement Under § 3.73(b)
___ Executed assignment and cover sheet
___ Information Disclosure Statement
___ Paper copy of sequence listing, pages ___ through ___
___ Sequence listing in computer readable form
___ Statement Under § 1.821(f)
___ Translation of the non-English application
___ Amendments to the PCT application under Article 19
___ Preliminary Amendment
___ Also enclosed: _____

Please address all future communications to:

Cathryn Campbell
CAMPBELL & FLORES LLP
4370 La Jolla Village Drive, 7th Floor
San Diego, California 92122
telephone: (858) 535-9001
facsimile: (858) 535-8949
USPTO CUSTOMER NO. 23601

Respectfully submitted,

Date: 17 January 2002


Calvin A. Fan
Registration No. 38,444
CAMPBELL & FLORES LLP
4370 La Jolla Village Dr., 7th Fl.
San Diego, California 92122



Rec'd PCT/PTO 24 JUN 2002

10031530 061503

He

PATENT

Our Docket: P-UX 5156

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)	Group Art Unit: Not yet known
Krupp et al.)	
)	Examiner: Not yet known
Serial No. 10/031,530)	
)	
Filed: January 17, 2002)	
)	I hereby certify that this correspondence is
)	being deposited with the United States
For: METHOD FOR THE)	Postal Service as first class mail in an
SPECIES-SPECIFIC)	envelope addressed to: Commissioner for Patents,
DETECTION OF ORGANISMS)	Washington, D.C. 20231, on June 18, 2002 .
)	
)	By <u><i>CAF</i></u>
)	Calvin A. Fan, Reg. No. 38,444

Commissioner for Patents
Washington, D.C. 20231

June 18, 2002
Date of Signature

Sir:

PRELIMINARY AMENDMENT

The Applicants respectfully submit the following amendment and remarks.

I. Amendment

Please cancel original claims 1 to 41 and add the following new claims:

42. A method for the species-specific detection of a prokaryote or eukaryote in a biological sample, comprising the steps of

(a) amplifying DNA from the biological sample using a nucleic acid amplification technique,

wherein the amplified region of the DNA is flanked by conserved sequences, and

wherein amplification primers are used that contain the conserved sequences;

- (b) adding a sequencing primer to the amplificates,

wherein the primer hybridizes to the amplified sequence within a region that is conserved in the prokaryote or eukaryote,

wherein the primer has been selected so that when a chain termination polymerization process is performed, then different elongates are obtained for different prokaryotes or eukaryotes,

- (c) performing a chain termination polymerization process to obtain an elongate;

wherein 1, 2, or 3 of the four possible dNTPs are used, or one of the four possible dNTPs is replaced by a chain termination NTP;

- (d) determining the length of the obtained elongate,

whereby the prokaryote or eukaryote is detected by matching the obtained elongate with a reference elongate for the prokaryote or eukaryote.

mendocina, *Pseudomonas syringae*, *Haemophilus influenzae*,
Haemophilus ducreyi and *Bacterioides spp.*, whereby a sepsis
inducer is detected.

68. The method of claim 67, further comprising enriching steps,

wherein the blood is introduced into a suitable quantity of
lysis buffer by mixing one part by volume of blood with 4
parts by volume of lysis buffer,

wherein the lysis buffer consists of 109.5 g (0.32 M)
sucrose, 1.221 g (10mM) Tris-HCl, 10 ml (1%) Triton
X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water
(pH 7.5);

wherein the mixture is then incubated at room temperature
for 5 minutes, and then layered over a suitable quantity of
a buoyant density solution in a centrifuge tube,

wherein 100 ml of the buoyant density solution consists
of 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000
ml), 49.2 ml Percoll with a density of 1.13 ± 0.005
g/ml, and 40.8 ml distilled water;

wherein the tube is centrifuged at 1500 x g at room
temperature for 30 minutes, and the pellet is preferably
then washed twice with 0.15 M NaCl solution at room
temperature, re-pelleted at 1500 x g, the bacteria pellet
resuspended in TE buffer,

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 8

wherein the TE buffer is 10 mM Tris-HCl (pH 8),
1mM EDTA); and

wherein the resuspension is digested with proteinase K at
56°C for 2 hours, and the proteinase K is then inactivated
at 95°C for 15 minutes,


whereby the lysate is used in amplifying step (a).

69. The method of claim 68, further comprising the step of
purifying the lysate.
70. The method of claim 68, wherein the lysate is further
purified by means selected from the group consisting of a
purification system based on a glass matrix for
decomposition of bacteria and elimination of inhibitors of
the amplification.
71. The method of claim 42, wherein the biological sample is
blood and the eukaryote is selected from the group
consisting of flagellates, amoebas, sporozoa and ciliates,
whereby protozoa are detected.
72. The method of claim 42, wherein the biological sample is
meat or meat juice and the prokaryote is salmonella, whereby
salmonella is detected.

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 9

73. The method of claim 42, wherein the biological sample is fish spawn and the eukaryote is a fish.
74. The method of claim 42, wherein the method is a medical diagnostic method.
75. A method for obtaining bacteria from a biological sample further containing buffer and nonbacterial cells, comprising the steps of
- (1) subjecting the nonbacterial cells to lysis using from 1 to 20 wt.% of a detergent selected from the group consisting of TWEEN (polyoxyethylene derivatives of sorbitan esters), TRITON (octylphenol ethylene oxide condensate) and CHAPS (3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate);
 - (2) incubating the sample at room temperature for 5 minutes;
 - (3) layering the mixture over a suitable quantity of a buoyant density solution with a density of 1.07 g/ml in a centrifuge tube; and
 - (4) centrifuging the tube at 1500xg at room temperature for 30 minutes;

whereby the bacteria are obtained in a pellet suitable for further use.



Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 10

76. The method of claim 42, further comprising, before amplification step (a), the steps of

- (1) subjecting the nonbacterial cells to lysis using from 1 to 20 wt.% of a detergent selected from the group consisting of TWEEN (polyoxyethylene derivatives of sorbitan esters), TRITON (octylphenol ethylene oxide condensate) and CHAPS (3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate);
- (2) incubating the sample at room temperature for 5 minutes;
- (3) layering the mixture over a suitable quantity of a buoyant density solution with a density of 1.07 g/ml in a centrifuge tube; and
- (4) centrifuging the tube at 1500xg at room temperature for 30 minutes to obtain a pellet.

77. The method of claim 75 or 76, wherein the pellet obtained is further washed twice with 0.15 M NaCl solution at room temperature and pelleted at 1500xg.

3

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 11

78. The method of claim 75 or 76, wherein a buoyant density solution is used, of which 100 ml consists of 10 ml (1.5 M) NaCl solution, 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml and 40.8 ml distilled water.
79. The method of claim 75 or 76, further comprising the step of subjecting the bacteria in the pellet to lysis.
80. The method of claim 79, wherein in the lysing step, the bacteria pellet is resuspended in TE buffer and digested with proteinase K at 56°C for 2 hours, and the proteinase K is then inactivated at 95°C for 15 minutes, whereby a lysate containing bacterial DNA is obtained.
81. A kit, comprising
- (a) lysis buffer, containing 1 to 20 wt.% detergent selected from the group consisting of TWEEN, TRITON or CHAPS;
 - (b) centrifuge tubes; and
 - (c) buoyant density solution of density 1.07 g/ml.
82. The kit of claim 81, wherein the lysis buffer consists of 109.5 g (0.32 M) sucrose, 1.221 g (10 mM) Tris-HCl, 10 ml (1%) Triton X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5).

- _____

Inventors: Krupp et al.
 Serial No. 10/031,530
 Filed: January 17, 2002
 Page 13

87. The kit of claim 81, further comprising

(a) vessels and components for a nucleic acid amplification technique, comprising

an amplification primer containing a sequence that is conserved in a preselected prokaryote or eukaryote;

(b) vessels and components for the implementation of at least one chain termination polymerization process, comprising

at least one sequencing primer that hybridizes with a region within the sequences to be amplified, which is conserved in the preselected prokaryote or eukaryote; and

(c) components for determination of the elongate length.

88. The kit of claim 86 or 87, wherein the sequencing primers are distinguishably labeled.

89. The kit of claim 86 or 87, wherein a sequencing primer has a length of 15 to 30 nucleotides.

10/031,530

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 14

90. The kit of claim 86 or 87, wherein a component for the implementation of the chain termination polymerization process comprises 1, 2, or 3 of the four possible dNTPs.
91. The kit of claim 86 or 87, wherein in a component for the implementation of the chain termination polymerization process, one of the four possible dNTPs is replaced by a chain termination NTP.
92. The kit of claim 91, wherein the chain termination NTP is selected from the group comprising a ddNTP, 3'-O-methyl-NTP and 3'-amino-NTP.
93. The kit of claim 86 or 87, wherein the components for determination of the elongate length are selected from the group consisting of devices or means for implementation of an electrophoretic method, a mass-spectroscopic method and fluorescence-correlation spectroscopy.
94. The kit of claim 86 or 87, wherein the kit is for the detection of prokaryotes, and comprises amplification primers having two highly conserved sequences from the rRNA region of the prokaryote.
95. The kit of claim 94, wherein the amplification primers are primer pair SEQ ID NO 1 and 2 or the primer pair SEQ ID NO 3 and 4.

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 15

96. The kit of claim **94**, wherein the kit comprises at least one sequencing primer that can hybridize with a conserved region of the rRNA region.
97. The kit of claim **96**, wherein a sequencing primer is selected from the group consisting of SEQ ID NO 5, 6, 7, 8, 9, 10 and 11.
98. The kit of claim **97**, wherein the kit comprises a mixture of the sequencing primers SEQ ID NO 5 and 6, a mixture of the sequencing primers SEQ ID NO 7 to 9, or a mixture of the sequencing primers SEQ ID NO 10 and 11.
99. The kit of claim **86** or **87**, wherein the kit is for the detection of sepsis inducers.

II. Remarks

The claims have been amended to replace the originally filed claims in the PCT application with new claims that conform better to U.S. practice. For example, plurals terms have been replaced with singular terms with the understanding that the term "comprising" anywhere in a claim encompasses the addition of further elements; lists of alternate limitations have been rewritten as Markush groups; multiple-dependent claims that depend from multiple-dependent claims have been reconfigured; and precatory wording from claims has been removed where it was not useful to define the invention.

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 16


In claim 42, rewritten from original claim 1, the terms "limited" and "preserved" have been replaced with "flanked" and "conserved" according to common scientific usage in the United States. Step (c) has been added to clarify that a chain termination polymerization process is actually performed to obtain the elongate used in step (d). Previous optional steps (d) and (e) in original claim 1 have been moved to dependent claim 46.

Original claim 2 has been divided into new Markush claim 43 and Markush claim 44.

Claims 3 to 7, 11 to 14 and 16 to 19, which originally depended from multiple previous claims in a European-style stacking arrangement, have been rewritten to depend from claim 42, the broadest base claim for methods.

Original 9 has been rewritten as claim 54 to clarify that the amplified DNA region is an rDNA. Claims 55, 56 and 57 have been added to specify the particular rRNA that is encoded by the amplified rDNA.

Original claim 10 has been rewritten as claim 58 to clarify that the DNA region is the amplified DNA region. Claims 59 and 60 have been added to specify the particular rRNA spacer region that is encoded by the rDNA.



As to original claim 12, now claim 62, three dependent claims 63, 64 and 65 have been added to specify specific combinations of sequencing primers and chain termination NTPs.

In claim 14, now claim 67, the names of the bacteria species have been italicized. Where more than one species is intended, the more common abbreviation *spp.* was used.

In claim 68, we have reformatted original claim 15 and added the composition of TE buffer, based on the disclosure in the specification on page 20, lines 8 and 9. The optional limitation for further purification has been moved to claim 70.

Claim 19 has been rewritten from a use claim to dependent method claim 74.

Claim 20 has been reformatted into claim 75 as a series of steps.

Claim 25, which previously made reference to both claims 20 to 24 and claims 1 to 14, has been rewritten as claim 76 to explicitly recite the steps in claim 1. Dependent claims 77 to 80 now depend, directly or indirectly, from both claim 75 and 76.

In claim 26, the term "centrifuge pipes" has been replaced with "centrifuge tubes" in claim 81 according to customary usage, and as supported in the specification on page 17, line 4.

Inventors: Krupp et al.
Serial No. 10/031,530
Filed: January 17, 2002
Page 18

Claim 28, which previously made reference to both claims 20 to 24 and claims 1 to 14, has been rewritten as claim 83 to explicitly recite the steps in claim 1.

In claim 31, the phrase "prokaryotes or eukaryotes in question to be detected" has been rewritten in claim 86 as "preselected prokaryote or eukaryote." Also, the optional limitations in (b) have been moved to claims 88 and 89.

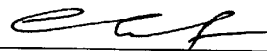
Claim 35, which previously made reference to both claims 31 to 34 and claims 25 to 29, has been rewritten as claim 87 to explicitly recite the steps in claim 31. Dependent claims 88 to 99 now depend, directly or indirectly, from both claim 86 and 87.

The Examiner is invited to call the undersigned attorney or Cathryn Campbell with any questions.

Respectfully submitted,

June 18, 2002

Date



Calvin A. Fan
Registration No. 38,444
Telephone No. (858) 535-9001
Facsimile No. (858) 535-8949

CAMPBELL & FLORES LLP
4370 La Jolla Village Drive
7th Floor
San Diego, California 92122
USPTO Customer No. 23601

WO 01/07648

Rec'd PCT/PTO 18 JUN 2002

PCT/EP99/05234

10/03/530

Method for the species-specific detection of organisms

The invention relates to a method for the species-specific detection of prokaryotes and eukaryotes and to kits for implementing said method. The invention relates especially to a method for the species-specific detection of sepsis inducers.

The reliable detection of a very wide range of organisms, especially microorganisms such as bacteria, plays an increasing role in medical microbiology and is often a prerequisite for the targeted treatment of infections in humans and animals. In this connection, Grtler et al., (Microbiology 142 (1996) 3-16) and Scheinert et al., J. Microbiol. Methods 26 (1996) 103-117) have proposed the use of the 16S-23S rDNA and/or rRNA spacer-region for the typing and identification of bacteria. The sequences of rRNAs have to date been used most often for the identification of microorganisms. In the genome of prokaryotic and eukaryotic organisms, the operons for ribosomal RNAs (rRNAs) are organized so that, initially, an

extensive precursor rRNA is transcribed. These precursor RNAs essentially contain the following components in a 5' - 3' direction:

Prokaryotes - ribosomal DNA includes sequences for:

5' - 16S rRNA - transcribed 16S/23S rRNA-spacer - 23S rRNA -
transcribed 23S/5S rRNA-spacer - 5S rRNA - 3'

Eukaryotes - ribosomal DNA includes sequences for:

5' - 18S rRNA - transcribed 18S/5.8S rRNA-spacer (ITS1) - 5.8S rRNA -
transcribed 5.8S/28S rRNA-spacer (ITS2) - 28S rRNA - 3'.

This general organization is present in all prokaryotes (*Bacteria* and *Archaea*) and eukaryotes (cf. e.g. B. Lewin, *Genes V*, Cell Press, Cambridge, Massachusetts/USA, 1994), with only very few exceptions, such as e.g. *Archaeon Thermoplasma acidophilum* (Achenbach-Richter et al., *System. Appl. Microbiol.* 10 (1988) 211-214) or *Helicobacter* (Tomb et al., *Nature* 388 (1997) 539-547) being known to date.

It has emerged that the lengths of the rRNA-spacers are extremely variable and within a well-defined group of microorganisms, such as e.g. mycoplasmas, the length alone is a suitable means for assignment of microorganisms to this group (e.g. mycoplasmas) and can also to some extent be used for the identification of individual species within this group.

In the method described by Grtler et al. (loc. cit.) and Scheinert et al. (loc. cit.) for the typing and identification of bacteria using the 16S-23S rRNA/rRNA spacer region, an amplification by polymerase chain reaction

(PCR-amplification) is initially carried out using suitable primers. The amplified spacers can then be gelelectrophoretically separated and detected using a suitable detection method, such as e.g. coloration with ethidium bromide or silver or by fluorescence detection. The position of the bands, i.e. the length of the amplified spacers, allows a preliminary conclusion regarding the microorganism/microorganism group detected in each case.

For certain detection, especially for the recognition of individual species, a secondary length difference is generally required after defined restriction-enzymatic treatment of the amplified spacers. The individual species within a microorganism group, following gelelectrophoretic separation and hybridization with species-specific oligonucleotide probes produce a characteristic band pattern.

These methods have the disadvantage that, for the detection of species-specific inducers, precise knowledge of the band pattern to be expected is required, and in addition an extremely large number of specific oligonucleotide probes must be available. The methods known to date in the state of the art are therefore not suitable for the routine detection of inducers in medical laboratories.

With regard to sepsis, a serious, acute infectious disease, which is caused by certain bacteria getting into the blood circulation, the additional problem also arises, that these bacteria go out from a local inflammatory focus and become spread in thrusts through the blood or lymphatic system. Because of this cyclical fluctuation, relatively large quantities of blood are required for bacterial septic detection (approx. 5 - 10 ml for aerobic and anaerobic blood culture in each case). With the means and methods currently available, this large sample volume is only manageable at considerable expense, as major contamination with foreign DNA (i.e. non-bacterial DNA)

leads to non-specific purification and thus to reduction of the specificity of a subsequent PCR. The large number of red blood corpuscles can further have an inhibiting effect on the PCR, and fairly large sample volumes are not generally suitable for commercially available kits, frequently resulting in blockage of the columns used for purification.

The task of the present invention is therefore to make available a method for the detection of organisms (prokaryotes and eukaryotes), e.g. in selected biological samples, which is simple to manage and suitable for routine use in medical laboratories. The method is further to have a high specificity with regard to a very large number of inducer groups, with it being possible to differentiate between individual species within these groups in a simple cost-effective manner, without the expense of experimentation. Finally, it is a task of the invention to make available a simple method enabling detection of microorganisms present only in low concentrations, as is the case e.g. at times in the cyclical development of sepsis.

The task is solved according to the invention by methods according to Claims 1 to 17 and 19 to 24 and kits according to Claims 25 to 40.

The invention thus relates to a method for the species-specific detection of prokaryotes or eukaryotes using nucleic acid amplification techniques, wherein an enrichment, concentration and/or multiplication of the prokaryotes or eukaryotes to be detected in a biological sample is possibly initially undertaken and/or the DNA present in the biological sample is separated and/or enriched. The amplification of the DNA is carried out using a nucleic acid amplification technique (NAT), wherein, using amplification primers which contain the sequences preserved for the

organisms concerned, a region of the DNA is amplified, which is flanked by the preserved sequences.

For implementation of the method according to the invention, any nucleic acid amplification techniques (NAT) familiar to the person skilled in the art can be used, preferably a NAT from the group consisting of PCR, bDNA, LCR, 3SR, SDA or NASBA.

In the next step, a sequencing primer is added to the (isolated) amplicates, said primer being hybridized with a region inside the amplified sequences, which is preserved with the prokaryotes or eukaryotes to be detected. The sequencing primer of this method step described as "minisequencing" is chosen so that, for the prokaryotes or eukaryotes to be detected, in each case different elongates are obtained if chain-termination polymerization is carried out, using 1, 2, or 3 of the four possible deoxyribonucleoside triphosphates (dNTPs) or replacing one of the four possible dNTPs by a chain-termination deoxyribonucleoside triphosphate (chain-termination-NTP). After determination of the growth in length obtained in each case (elongates; i.e. number of bases by which the amplicates have been elongated), which the products of chain-termination polymerization have compared with the amplicates, these values are compared with the elongates, which are to be expected for the organisms in question due to their sequence. A specific prokaryote or eukaryote present in the sample is detected if the observed (achieved) elongation and expected elongation match.

In individual cases it may happen that several of the organisms present in the sample produce the same elongates (i.e. the same growth in length in comparison with the amplicates) if a single sequencing primer is used. Then, if necessary, one or more further sequencing primers is/are used, which hybridize(s) with a range/ranges within the amplified sequences, which is/are preserved in the case of the prokaryotes or eukaryotes to be

detected, wherein the further sequencing primer(s) is/are chosen so that different elongates are obtained in each case for the prokaryotes or eukaryotes to be detected if chain-termination polymerization is carried out, wherein 1, 2 or 3 of the four possible dNTPs are used or one of the four possible dNTPs is replaced by a chain-termination NTP. After determination of the growth in length obtained in each case (elongates; number of bases by which the amplicates have been elongated), which the products of chain-termination polymerization have compared with the amplicates, these values are compared with the elongates which are to be expected for the organisms in question due to their sequence. A prokaryote or eukaryote present in the sample is detected if, with use of the different sequencing primers, experimentally obtained elongation values (experimentally determined elongation pattern) match the elongation values to be expected for this organism (expected elongation pattern).

For the species-specific detection of organisms in biological samples, precisely so many different sequencing primers thus have to be used, until different elongation patterns are obtained for all organisms possibly present in the sample. In the example section this is illustrated with reference to the determination of sepsis inducers.

The sequencing primers used in the chain termination polymerization preferably have a length of 15 to 30 nucleotides. Possible chain terminator NTPs are, e.g. dideoxyribonucleoside triphosphates (ddNTPs), 3'-O-methyl-NTPs, 3'-amino-NTPs and the like.

According to a special embodiment of the invention the method can be implemented using several sequencing primers, wherein, in the chain termination reaction of the four possible dNTPs one is replaced by a chain termination NTP (e.g. ddA), wherein the polymerization takes place in the

simultaneous presence of all the sequencing primers, wherein the sequencing primers each bear different labelling, making it possible to differentiate between the elongates in each case.

The length of the elongates can be determined by methods known to the person skilled in the art, such as e.g. by means of electrophoretic methods, by mass spectrometric detection or, e.g. by fluorescence correlation spectroscopy (FCS) or comparable methods. In this connection it is advantageous preferably to mark the sequencing primer. If several sequencing primers are used, these should be marked differently, especially if the polymerization processes are carried out in a single reaction vessel using only one terminator (e.g. ddA). Suitable markings, e.g. fluorescence labels, or labellings differing in mass, are well known to the person skilled in the art.

According to an especially preferred embodiment of the invention the primer elongates are mass spectroscopically detected, MALDI-TOF spectrometry (Matrix-assisted laser desorption ionization time of flight spectrometry, cf. e.g. Fu et al., Nature Biotechnol. 16 (1998) 381-384)) having proved especially advantageous, allowing detection of up to 2000 nucleotides or more.

In this connection, "multiplex analysis", i.e. the simultaneous analysis of several sequencing primers with different target sequences, is advantageous. For this purpose the elongation reactions with differently labelled sequencing primers are carried out either in a reaction vessel using a single terminator (Alternative A) or in different reaction vessels using different terminators (Alternative B), with the products of the (parallel) elongation reactions being united for the analysis in Alternative B. The necessary differentiation of the elongation products is

possible by labelling the oligonucleotides with:

- (a) 5'-terminal addition sequences, "tails" of different lengths (usable in all detection methods),
- (b) different 5'-terminal additions which drastically change the development (in the case of standard polyacrylamide gelelectrophoresis, or highly-dissolving gelelectrophoresis such as plate gel, capillary or array capillary gelelectrophoresis) or molecular weight (MALDI-TOF). Examples of such additions are polyethylene glycol chain, cholesterol derivatives etc.,
- (c) different fluorescence dyes (in the case of standard polyacrylamide gelelectrophoresis, combined with a fluorescence scanner; routinely in the case of highly-dissolving gelelectrophoresis, in principle also possible in the case of mass spectroscopic analysis, wherein overlapping elongation patterns can also be differentiated by means of characteristic molecular weight values and the extremely high dissolving capacity).

The method according to the invention is not in principle subject to any methodical restrictions to a specific group of organisms. A prerequisite for the certain (re-)recognition and differentiation of organisms is only that the target sequences (e.g. rRNA, rDNA) of the organisms to be detected are known. The method of organism determination according to the invention based on universal sequences, i.e. sequences essentially present in all organisms (such as e.g. ribosomal sequences) is therefore suitable for differential detection of definite groups of organisms of all species of prokaryotes and eukaryotes (bacteria, fungi, plants and animals).

Within the context of the present invention, the term "prokaryotes" means

representatives of the group of *Bacteria* and *Archaea* and includes all species falling within these groups. The term "eukaryotes" includes both single- and multi-cell organisms, such as, e.g. amoebas, trypanosomes, plasmodia, yeasts, single- and multi-cell parasites, as well as plants and animals, and covers all species falling within these groups.

Within the framework of the present invention, in principle all variable sequences flanked by preserved areas, which are present in all the organisms (to be detected) can be considered as target sequences. For example it is possible to utilize the variable rDNA sequences including rRNA spacers as target sequences. In addition however, other species-specific genes or gene sections can also be used as a starting point, e.g. cytochrome b gene from mitochondria (cf. Irwin et al., *J. Mol. Evol.* 32 (1991) 128-144), which can be used for the species-specific detection of eukaryotes.

The method according to the invention can be used in a very wide range of areas. The (micro-)organisms mentioned in the following examples of use are however only representative of other representatives of the ranges of organism in question (prokaryota = bacteria, eukaryota = single- and multi-cell fungi, plants and animals) as differentiated in the biological classification system:

- a) Detection of sepsis inducers and other bacterial inducers (pathogens) in humans, animals and plants and in cell cultures thereof;
- b) Detection of single-cell organisms (protozoa: flagellates, amoebas, sporozoa, ciliates) as causes of e.g. intestinal diseases in humans and animals (especially farm animals);
- c) Detection of pests, contaminants, decay inducers and pathogens in

agriculture, animal husbandry, the food industry and in seeds: here again, defined groups of organisms from all the groups in the biological classification system (see above) can be differentiated and are expressly included, especially from the groups of bacteria, protozoa, fungi and arthropods;

- d) Detection of helminths (worms) as causes of intestinal diseases (e.g. diarrhoea);
- e) Detection of fungi as inducers of fungal diseases in humans, animals and plants;
- f) Determination of species, breeds and origin in animal husbandry and plant cultivation, species breeding and agriculture, fisheries (e.g. sampling when checking catch quotas) and in food inspections;
- g) Bio-diversity checks for defined groups of organisms such as, e.g. fresh- or saltwater fish including their larval stages in Europe or other geographically delimited regions (e.g. for determination of stock numbers for catch quotas etc.), determination of main organisms for expert reports on water or soil.

The method of the present invention can in particular be used universally in medical diagnostics, e.g. to clarify the question of which (groups of) inducers are present when a specific disease symptom is observed. Thus, for example, the causes of the following medically relevant disease symptoms can be determined by means of the method according to the invention:

- fever as a result of bacterial (sepsis), parasitic (e.g. malaria) or fungal inducers (e.g. candida) in the blood;

- Inflammation of the brain or meningitis, i.e. headache, stiff neck, clouding of the consciousness due to bacterial (meningococcus, haemophilus influenza, pneumococcus, tubercular bacillus, E. coli, Listeria monocytogenes) parasitic (e.g. toxoplasmosis) or fungal infections (e.g. Cryptococcus neoformans);
- Infections of the respiratory tract, i.e. cough, phlegm, shortness of breath etc. due to bacterial (e.g. pneumococcus, chlamydia, mycoplasma), parasitic or fungal infections (e.g. Pneumocystis carinii);
- Eye infections, i.e. watering eyes, possibly pus, clouded vision etc. Possible causes may again include a number of bacterial (e.g. chlamydia, gonorrhoea, Staphylococcus aureus) or parasitic inducers (e.g. Toxoplasma gondii, Onchocerca volvulus etc.);
- Diarrhoea and weight loss etc. as a result of bacterial (e.g. salmonella, yersinia, campylobacter, E. coli, vibrio, clostridia, bacillus) parasitic (e.g. amoebas, giardia, cryptosporidia) or fungal infections (candida);
- Pain on micturition, haematuria etc. as a symptom of urinary tract infection, e.g. caused by E. coli, staphylococcus, further enterobacteria such as Proteus mirabilis as bacterial inducers, candida as a fungus, or schistosomes as parasitic inducers. This group also includes the inducers transmitted primarily sexually, such as e.g. chlamydia, gonorrhoea, syphilis, mycoplasmas etc.
- Skin infections, i.e. reddening of the skin, itching, blistering etc. due to fungal infections with dermatophytes such as e.g. trichophyton, epidermophyton and microsporon, bacterial infections (e.g.

staphylococcus or Steptococcus pyogenes) or parasitic diseases, e.g. leishmaniasis.

By use of the method according to the invention, inducers can be detected species-specifically, making possible treatments which are more targeted and thus often have fewer side effects than is the case with broader-based therapies.

Within the context of the present invention, the term "biological samples" means all types of samples in which a restricted, well-defined group of prokaryotes or eukaryotes may be present. When the method according to the invention is used in medical diagnostics, the biological sample can be e.g. blood, faeces, swabs etc. (e.g. detection of sepsis inducers in blood, detection of protozoa in blood or faeces etc.). In the detection of food contaminants, plant pests and the like, animal or plant tissue or fluids, e.g. meat or meat juice, can generally be used as a sample (e.g. detection of salmonella in meat, detection of plant pests in seeds). Environmental samples, e.g. soil or water samples also come into consideration. In the case of fish stock analyses or, e.g. to determine species in fish farming and fisheries biology, spawn or a sample of plankton may also be used as a biological sample.

The person skilled in the art is able to select the biological samples for the species-specific detection of organisms so that the group of organisms that may be present in the sample is limited and well-defined. Thus, for example, when clarifying a sepsis, the inducers responsible for this disease are known, so that, on examination of a blood sample, the clinical suspicion of sepsis can be confirmed, if, by using the method according to the invention, one or more of the bacteria listed in Table 1 is detected.

According to a special embodiment of the invention, a method for detecting sepsis inducers is thus made available, wherein the biological sample is blood and the organisms are selected from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Neisseria meningitidis*, *Escherichia coli*, *Enterobacter spec.*, *Proteus spec.*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas mendocina*, *Pseudomonas syringae*, *Haemophilus influenzae*, *Haemophilus ducreyi* and *Bacterioides spec.* The method is preferably implemented, by introducing blood from the patient into buffer and subjecting the human cells to lysis, e.g. using from 1 to 20 wt.% Tween® (polyoxyethylene derivatives of sorbitan esters), Triton® or 3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate (CHAPS). Blood introduced into lysis buffer by mixing one part by volume of blood with 4 parts by volume of lysis buffer is especially preferred, wherein the lysis buffer consists of 109.5 g (0.32 M) sucrose, 1.221 g (10mM) Tris-HCl, 10 ml (1%) Triton X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5), the mixture is then incubated at room temperature for 5 minutes, and then layered over a suitable quantity of a buoyant density solution with a density of 1.07 g/ml in a centrifuge tube and, at 1500 g, centrifuged at room temperature for 30 minutes, whereby the bacteria are obtained in a pellet (enriched). According to a preferred embodiment of the invention, a buoyant density solution is used, of which 100 ml consists of 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml and 40.8 ml distilled water.

The pellet is preferably then washed twice with 0.15 M NaCl solution at room temperature, pelleted at 1500 g, and the bacteria pellet re-suspended in TE buffer (10 mM Tris-HCl (pH 8), 1 mM Na₂EDTA) is subsequently digested with proteinase K at 56°C for 2 hours; the proteinase K is then

inactivated at 95°C for 15 minutes, and the lysate obtained, which contains the bacterial DNA, is either used directly in a nucleic acid-amplification technique, or further purification is first carried out using e.g. a purification system based on a glass matrix (glass milk) for decomposition of bacteria, and/or elimination of inhibitors of the amplification.

With regard to the buoyant density solution, instead of Percoll, sucrose or Ficoll can be used. The density of the buoyant solution should in each case be 1.07 g/ml.

Then - as indicated above - a NAT generally known to the person skilled in the art is carried out, followed by chain termination polymerization.

According to a preferred embodiment of the invention, in the detection of sepsis inducers, the primer pair according to SEQ ID NO 1 and 2 or the primer pair according to SEQ ID NO 3 and 4 are used as amplification primer, the primer pair according to SEQ ID NO 1 and 2 being especially preferred. For the chain termination polymerization, at least one sequencing primer from the group consisting of SEQ ID NO 5, 6, 7, 8, 9, 10 and 11 are used (cf. also D.J. Lane in "Nucleic Acid Techniques in Bacterial Systematics", John Wiley & Sons 1991, pp. 115-175). Three chain termination polymerizations are especially preferably carried out, one reaction in the presence of the sequencing primer according to SEQ ID NO 5 and 6 (preferably with ddA as terminator), one reaction with the primers according to SEQ ID NO 7, 8 and 9 (preferably with ddG as terminator), and one reaction in the presence of the primers according to SEQ ID NO 10 and 11 (preferably with ddA as terminator).

Advantages of the method of the present invention compared with length determination of the PCR fragments in an agarose gel with and without enzymatic restriction digestion include increased specificity (detection

certainty) in particular. Use of an internal primer and its elongation (with minisequencing, see above) results in a specific recognition of the PCR fragment and thus increased certainty within the meaning of an clear statement compared with the mere length determination of PCR fragments without an identity check as, e.g., in the case of Grtler et al. 9cf. Microbiology 142 (1996) 3-16; WO96/19585).

Approximately the same time is required to carry out the NAT (e.g. PCR) and the chain termination polymerization of the method according to the invention, as for a traditional method with PCR and subsequent enzyme treatment (cf. e.g. Grtler, loc. cit.) However, as the detection/length determination of the elongates can be automated (e.g. by using MALDI-TOF spectrometry), the final analysis can be carried out within seconds or minutes. In contrast, traditional gel analysis can, by its nature, only be automated with difficulty and is thus also more cost-intensive than the solution made available according to the invention.

Moreover, the special advantage of the method according to the invention lies in the fact that, surprisingly, a simple and generally applicable method is made available, whereby species-specific detection of organisms is possible which - depending on the type of (biological) sample - belong to a limited, well-defined group of prokaryotes or eukaryotes.

The method developed according to a partial aspect of the present invention for the isolation and/or enrichment of bacterial DNA (and also for the preparation/concentration of bacteria) can also be used in other areas of application as an isolated enrichment method - especially of biological samples from the group consisting of blood or blood products, meat juice, milk (waste) water or any other liquid that may contain bacteria. As

already mentioned, the method is characterized in that the biological sample is possibly introduced into buffer and the non-bacterial cells subjected to lysis, e.g. using from 1 to 20 wt.% Tween® (polyoxyethylene derivatives of sorbitan esters), Triton® or 3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate (CHAPS). Alternatively, the above-mentioned preferred lysis buffer can be used in the same or similar proportions by volume. After incubation at room temperature for 5 minutes, the mixture is then layered over a suitable quantity of a buoyant density solution (see above) in a centrifuge tube. In the case of further processing including proteinase K digestion and subsequent proteinase K inactivation, the procedure indicated above for the example of sepsis inducers is preferably followed. The lysate obtained can e.g. either be used directly in a nucleic acid-amplification technique, or further purification is first carried out using e.g. a purification system based on a glass matrix for decomposition of bacteria, and/or elimination of inhibitors of the amplification.

The advantages of the enrichment method according to the invention lie in the fact that the separation of a large quantity of DNA, e.g. originating from blood cells, is made possible. It is also advantageously possible to remove substances originating from the blood (haemoglobin etc.), which can inhibit the implementation of nucleic acid amplification techniques.

Also within the framework of the present invention, kits are made available for implementation of the above-mentioned method.

A kit for implementation of the method for preparation and/or concentration of bacteria from biological samples - especially from blood or blood products, meat juice, milk, (waste) water or any other liquid that may contain bacteria - contains

- a) lysis buffer containing 1 to 20 wt.% Tween® (polyoxyethylene derivatives of sorbitan esters), Triton® or 3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate (CHAPS),
- b) centrifuge tubes,
- c) buoyant density solution with a density of 1.07 /ml, over which the biological sample is layered.

The kit's lysis buffer consists especially preferably of 109.5 g (0.32 M sucrose, 1.221 g (10 mM) Tris HCl, 10 ml (1%) Triton x-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5).

The kit's buoyant density solution preferably consists of 10 ml 1.5 M NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml, 40.8 ml distilled water (per 100 ml buoyant density solution).

The kits for implementation of the method for isolation and/or enrichment of bacterial DNA can possibly contain further components which are required or suitable for (further) sample preparation. Thus the kits contain in addition components for decomposition of the bacteria and possibly for DNA purification (especially for the elimination of amplification inhibitors) or the like, such as e.g. components of commercial purification systems based on a glass matrix (i.e. QIAamp DNA kit). In addition, such a kit will preferably contains Proteinase K for the decomposition of bacteria.

A kit for implementation of the method of species-specific detection of prokaryotes or eukaryotes from biological samples or environmental samples contains - possibly in addition to the components of one of the above-

mentioned kits for implementation of the DNA processing and enrichment method - the following components:

- a) Vessels and components for carrying out a NAT, including the amplification primer, wherein the amplification primer contains sequences which are preserved in the case of the particular prokaryotes or eukaryotes to be detected,
- b) Vessels and components for carrying out one or more chain termination polymerizations, including one or more (possibly labelled/possibly differently labelled) sequencing primers (preferably with a length of 15 to 30 nucleotides), which hybridize with a range within the sequences to be amplified, which is preserved in the case of the prokaryotes or eukaryotes to be detected.

For implementation of the chain termination polymerization, the kit contains either 1, 2, or 3 of the four possible deoxyribonucleoside triphosphates (dNTPs), or all four possible dNTPs, with one dNTP being replaced by a chain-termination deoxyribonucleoside triphosphate (such as e.g. a ddNTP, 3'-O-methyl-NTP, 3'-amino-NTP or the like).

The kit may possibly further contain components for determination of the elongate length (e.g. for carrying out electrophoresis) and/or corresponding components, that are necessary or useful for preparation of corresponding methods with which the elongate length can be determined. Such components will be clear to the person skilled in the art from the above description of the method for the species-specific detection of prokaryotes or eukaryotes, to which reference is expressly made in this connection.

According to a special embodiment of the invention, the kit is e.g. a kit for the detection of prokaryotes, especially sepsis inducers, which contains as amplification primer two highly preserved sequences from the rRNA region, preferably the amplification primers according to SEQ ID NO 1 and 2 or according to SEQ ID NO 3 and 4 (the primary pair according to SEQ ID NO 1 and 2 being especially preferred), and/or at least one sequencing primer which hybridizes with one preserved area of the rRNA region, preferably at least one sequencing primer from the group consisting of SEQ ID NO 5, 6, 7, 8, 9, 10 and 11 (mixtures or blends of the sequencing primers according to SEQ ID NO 5 and 6, 7 to 9 and 10 and 11 in a single container or separate containers being especially preferred).

The present invention is explained in more detail below with reference to examples.

Example 1

Method for the enrichment of microorganisms in biological samples:

In this example bacteria from blood were prepared/enriched. For this purpose blood was first placed in lysis buffer consisting of 109.5 g (0.32 M) sucrose, 1.221 g (10 mM) Tris-HCl, 10 ml (1%) Triton X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5), one part blood being mixed with four parts lysis buffer (e.g. 3 ml blood with 12 ml lysis buffer) and incubated at room temperature for 5 minutes.

The blood-lysis buffer mixture was then poured onto a buoyant solution (5 ml) and the whole solution of 1500g was centrifuged at room temperature

(RT) for 30 minutes. 100 ml of the buoyant density solution is made up as follows: 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 (+/- 0.005 g/ml), and 40.8 ml distilled water. Instead of Percoll, sucrose or Ficoll can be used. The density of the buoyant solution should in each case be 1.07 g/ml.

The pellet in which the bacteria were to be found was then washed twice with 0.15 M NaCl solution at RT and pelleted at 1500 g.

Proteinase K digestion of the bacterial pellet re-suspended in TE buffer (10 mM Tris HCl (pH 8), 1 mM Na₂EDTA) then took place at 56°C for two hours, and the proteinase K was then inactivated at 95°C for 15 minutes. The lysate obtained was then used in the amplification. Alternatively commercial purification systems based on a glass matrix (i.e. QIAamp DNA kit) can be used for the decomposition of bacteria and for the elimination of inhibitors of the amplification.

Example 2

Detection of microorganisms by PCR-supported amplification and subsequent elongation of the amplicates:

The bacterial DNA obtained in Example 1 was subjected to PCR amplification and the amplicates obtained were then used in a chain termination polymerization process. These methods and the conditions under which these methods were carried out are well known to the person skilled in the art (cf. e.g. Garcia-Pichel et al., Arch. Microbiol. 169 (1998) 469-482; Cha et al., PCR Methods and Application 3 (1993) pp. 18-29, (Manual Supplement); Tracy et al., Bio Techniques 11 (1) (1991) 68-75).

A. Nucleic acid amplification:

Using the PCR, the 16S rRNA regions were amplified with two highly preserved primers. For this purpose a 16S-5' terminal (SEQ ID NO 1) and a 16S-3' terminal primer (SEQ ID NO 2) were combined, with one of the two PCR primers (in this case the 16S-5' terminal primer) containing a derivate suitable for immobilization, e.g. biotin.

Instead of the 16S rRNA, the 16S-23S-rRNA spacer can also be amplified, possibly with the primers according to SEQ ID NO 3 (16S-proximal primer) and SEQ ID NO 4 (23S-proximal primer).

It is obvious to the person skilled in the art that the result of the PCR is not fundamentally influenced by modification of the oligonucleotides (wobbles, mismatches, biotin-, digoxigen- or fluorescence labelling etc.).

The amplified rRNA regions were subsequently subjected to an elongation process.

B. Elongation of the amplificates:

For elongation of the amplified spacer sequences, oligonucleotides were used as sequencing primers, which are universally preserved or at least present in all target organisms within the group to be detected. After elongation with a polymerase - omitting one of the four natural nucleoside triphosphates or in the presence of a terminator nucleoside triphosphate (e.g. ddNTP) - products of different characteristic lengths were obtained.

For the elongation the following sequencing primers present in each case were used, which are complementary to 16S rRNA sequences, which are universally present in all bacteria (bacteria and archaea) (cf. also D.J. Lane op. cit.):

109r: SEQ ID NO 5 (109r1) and SEQ ID NO 6 (109r2)

685r: SEQ ID NO 7 (685r1) SEQ ID NO 8 (685r2),

SEQ ID NO 9 (685r3).

For the elongation process, two further differently-labelled sequencing primers were used, 1475r^s (SEQ ID NO 10) and 1475r^h (SEQ ID NO 11).

1475r^s is - within the sepsis inducers - specific for the group of gram-positive cocci. 1475r^h is - within the sepsis inducers - specific for the group *Haemophilus*.

The corresponding 16S rRNA sequences were selected, as very divergent sequences are present directly adjacent, i.e. sequences that can be used for a species-specific differentiation of microorganisms (bacteria).

For sequencing primer 109r the nucleoside triphosphates dGTP, dCTP, dTTP were used either alone or with the addition of ddATP (as indicated in Table 1). If dGTP, dCTP, and dTTP are used either alone, the values indicated in Table 1 are to be reduced by one nucleotide unit.

For sequencing primer 685r the nucleoside triphosphates dATP, dCTP, dTTP were used either alone or with the addition of ddGTP (as indicated in Table 1). If dGTP, dCTP, and dTTP are used either alone, the values indicated in Table 1 are to be reduced by one nucleotide unit.

For sequencing primer 1475r the nucleoside triphosphates dGTP, dCTP, dTTP were used either alone or with the addition of ddATP (as indicated in Table 1). If dGTP, dCTP, and dTTP are used either alone, the values indicated in Table 1 are to be reduced by one nucleotide unit.

The elongation reactions were carried out under conditions generally known

to the person skilled in the art (cf. e.g. Fu et al., loc. cit.).

The oligonucleotide elongates obtained with the above-mentioned primers are indicated in Table 1 below.

In the present example, the elongates were detected by fluorescence detection or mass spectrometry. Clear differentiation between *Pseudomonas aeruginosa* (elongates of 10 and 4 bases) and *Haemophilus influenzae* (elongates of 6 and 3 bases) was possible (see Table 1).

The set of data obtained using the primers 109r and 685r according to Example 2 did not initially make it possible to clearly differentiate between *Staphylococcus aureus* and *Staphylococcus epidermis*. This is however indispensable and was achieved by the additional use of the group-specific primer 1475r^a. Differentiation of the two *Haemophilus* species was also possible by using the primer 1475r^b (cf. Table 1).

The results represented in Table 1 show that, when the sequence-specific primers are used, characteristic elongates are obtained, which allow differentiation between different inducers.

The species listed in Table 1 cover ca. 80% of all typical sepsis cases (Geerdes-Fenge et al. (1994) Chemother. J. 3, 131-143). Detection of sepsis is thus possible using only three sequence-specific primers, preferably 109r, 685r and 1475r.

Table 1: Sequence-specific oligonucleotide elongates within 16S rRNA regions

Species	Primer 109r ^a , ddA terminator length of the elongate (bases)	Primer 685r ^b , ddG terminator length of the elongate (bases)	Primer 1475r ^c , ddA terminator length of the elongate (bases)
Gram-positive Cocci			
<i>Staphylococcus aureus</i>	9	5	6 ^s
<i>Staphylococcus epidermidis</i>	9	5	5 ^s
<i>Streptococcus pneumoniae</i>	5	5	7 ^s
<i>Streptococcus pyogenes</i>	7	5	62 ^s
<i>Enterococcus faecalis</i>	8	2	
Gram-negative Cocci			
<i>Neisseria meningitidis</i>	1	2	
Enterobacteria: gram-negative rods			
<i>Escheria coli</i>	6	5	
<i>Escheria spec.</i>	13	5	
<i>Proteus spec.</i>	13	3	
Gram-negative flagellated Pseudomonads			
<i>Pseudomonas aeruginosa</i>	10	4	
<i>Pseudomonas fluorescens</i>	12	19	
<i>Pseudomonas mendocina</i>	9	4	
<i>Pseudomonas syringae</i>	13	4	
Pasteurellaceae: gram-negative unflagellated rods			
<i>Haemophilus influenzae</i>	6	3	5 ^h
<i>Haemophilus ducreyi</i>	6	3	7 ^h
Obligate anaerobic gram-negative rods			
<i>Bacteroids spec.</i>	13(14)**	6	

^a) Mixture of 109r1 and 109r2, ^b) mixture of 685r1, 685r2 and 685r3, ^c) mixture of 1475r^s and 1475r^h

(14)**: Only one isolate in this relatively heterogeneous species produces this elongate length.

Claims:

1. Method for the species-specific detection of prokaryotes or eukaryotes using nucleic acid amplification techniques, characterized in that the following steps are carried out:
 - a) Amplification of the DNA from biological samples using a nucleic acid amplification technique,

wherein a region of the DNA is amplified, which is limited by preserved sequences, using amplification primers which contain the preserved sequences,
 - b) Addition of a sequencing primer to the amplicates, which hybridizes with a range within the amplified sequences, which is preserved in the case of the prokaryotes or eukaryotes to be detected, wherein the sequencing primer is selected so that different elongates are obtained for the prokaryotes or eukaryotes to be detected in each case, if a chain termination polymerization process is carried out, wherein 1, 2, or 3 of the four possible dNTPs are used, or one of the four possible dNTPs is replaced by a chain termination NTP.
 - c) Determination of the length of the elongates obtained,

wherein the prokaryotes or eukaryotes are detected by matching the elongates obtained with the elongates to be expected for the prokaryotes or eukaryotes in question based on their sequence,
 - d) wherein one or more further sequencing primers are possibly used, which hybridize with one region/regions within the amplified

sequences, which are preserved in the case of the prokaryotes or eukaryotes to be detected,

wherein the further sequencing primer(s) is/are chosen so that, for the prokaryotes or eukaryotes to be detected, different elongates are obtained in each case, if a chain termination polymerization process is carried out, wherein 1, 2, or 3 of the four possible dNTPs are used, or one of the four possible dNTPs is replaced by a chain termination NTP,

and wherein, by the use of the further sequencing primer/sequencing primers for the prokaryotes or eukaryotes to be detected, different elongate patterns are obtained in each case,

e) Determination of the length of the elongates obtained,

wherein the prokaryotes or eukaryotes are detected in that the elongate patterns obtained by the use of several sequencing primers match with the elongate patterns to be expected for the prokaryotes or eukaryotes to be detected on the basis of their sequence.

2. Method according to Claim 1, characterized in that before the amplification, enrichment, concentration and/or multiplication of the prokaryotes or eukaryotes and/or isolation and/or enrichment of the DNA is carried out.
3. Method according to Claim 1 or 2, characterized in that a nucleic acid amplification technique is chosen from the group consisting of PCR, bDNA, LCR, 3SR, SDA or NASBA.

4. Method according to Claims 1 to 3, characterized in that several sequencing primers are used and, during the chain termination reaction of the four possible dNTPs, one is replaced by a chain termination NTP, wherein the polymerization is carried out in the presence of all the sequencing primers, wherein the sequencing primers in each case bear different labellings.
5. Method according to Claims 1 to 4, characterized in that the sequencing primer(s) has/have a length of 15 to 30 nucleotides.
6. Method according to Claims 1 to 5, characterized in that the chain termination NTP is dideoxyribonucleoside triphosphate (ddNTP), 3'-O-methyl-NTP or 3'-amino-NTP.
7. Method according to Claims 1 to 6, characterized in that the length of the elongates is determined by means of electrophoretic methods, by mass-spectrometric detection or by fluorescence-correlation spectroscopy.
8. Method according to Claim 7, characterized in that the length of the elongates is determined by matrix-assisted laser desorption ionization time of flight spectroscopy (MALDI-TOF spectrometry).
9. Method according to Claims 1 to 8, characterized in that the DNA region is the amplified rDNA spacer region.
10. Method according to Claim 9, characterized in that the DNA region is the rRNA spacer region.
11. Method according to Claims 1 to 9, characterized in that the primer pair according to SEQ ID NO 1 and 2 or the primer pair according to SEQ ID NO 3 and 4 is used as amplification primers.

12. Method according to Claims 1 to 11, characterized in that at least one chain termination polymerization process is carried out and the sequencing primer(s) selected from the group consisting of sequencing primers according to SEQ ID NO 5, 6, 7, 8, 9, 10 and 11.
13. Method according to Claims 1 to 8, characterized in that it is a method for the species-specific detection of eukaryotes and the amplified DNA region is the cytochrome b gene from mitochondria.
14. Method according to Claims 1 to 12, characterized in that it is a method for the detection of sepsis inducers, wherein the biological sample is blood and the organisms are chosen from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Neisseria meningitidis*, *Escherichia coli*, *Enterobacter spec.*, *Proteus spec.*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas mendocina*, *Pseudomonas syringae*, *Haemophilus influenzae*, *Haemophilus ducreyi* and *Bacterioides spec.*
15. Method according to Claim 14, characterized in that blood is introduced into a suitable quantity of lysis buffer by mixing one part by volume of blood with 4 parts by volume of lysis buffer, wherein the lysis buffer consists of 109.5 g (0.32 M) sucrose, 1.221 g (10mM) Tris-HCl, 10 ml (1%) Triton X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5), the mixture is then incubated at room temperature for 5 minutes, and then layered over a suitable quantity of a buoyant density solution in a centrifuge tube wherein 100 ml of the buoyant density solution is consists of 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml, and 40.8 ml distilled water and, at 1500 g, centrifuged at room temperature for 30 minutes, the pellet is preferably then washed twice with 0.15 M NaCl

solution at room temperature, pelleted at 1500 g, and the bacteria pellet re-suspended in TE buffer is subsequently digested with proteinase K at 56°C for 2 hours and the proteinase K is then inactivated at 95°C for 15 minutes, and the lysate obtained is then either used directly in a nucleic acid-amplification technique, or further purification is first carried out using e.g. a purification system based on a glass matrix for decomposition of bacteria, and/or elimination of inhibitors of the amplification.

16. Method according to Claims 1 to 10, characterized in that it is a method for the detection of protozoa, wherein the biological sample is blood and the eukaryotes are chosen from the group consisting of flagellates, amoebas, sporozoa and ciliates.
17. Method according to Claims 1 to 11, characterized in that it is a method for the detection of salmonella, wherein the biological sample is meat or meat juice and the prokaryotes are salmonella.
18. Method according to Claims 1 to 10, characterized in that the biological sample is fish spawn and the eukaryotes are fish.
19. Use of a method according to Claims 1 to 15 in medical diagnostics.
20. Method for the preparation and/or concentration of bacteria, characterized in that a biological sample is possibly introduced into buffer and non-bacterial cells present subjected to lysis using from 1 to 20 wt.% Tween® (polyoxyethylene derivatives of sorbitan esters), Triton® or 3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate (CHAPS) and incubation at room temperature for 5 minutes, the

mixture is then layered over a suitable quantity of a buoyant density solution with a density of 1.07 g/ml in a centrifuge tube and, at 1500 g, centrifuged at room temperature for 30 minutes, whereby the bacteria are obtained in a pellet and/or enriched.

21. Method according to Claim 20, characterized in that the pellet obtained is further washed twice with 0.15 M NaCl solution at room temperature, pelleted at 1500 g.
22. Method according to Claim 20 or 21, characterized in that a buoyant density solution is used, of which 100 ml consists of 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml and 40.8 ml distilled water.
23. Method for the isolation and/or enrichment of bacterial DNA, characterized in that a method according to any one of Claims 20 to 22 is implemented and the bacteria in the pellet are then subjected to lysis.
24. Method according to Claim 23, characterized in that lysis is carried out, wherein the bacteria pellet is re-suspended in TE buffer and digested with proteinase K at 56°C for 2 hours and the proteinase K is then inactivated at 95°C for 15 minutes whereby a lysate is obtained, in which the bacterial DNA is contained.
25. Method for the species-specific detection of prokaryotes or eukaryotes using nucleic acid amplification techniques, characterized in that a method according to Claims 20 to 24 and subsequently a method according to Claims 1 to 14 is carried out.

26. Kit for the implementation of a method according to Claims 20 to 22, characterized in that it contains:

- a) lysis buffer containing 1 to 20 wt.% Tween® (polyoxyethylene derivatives of sorbitan esters), Triton® or 3-[N-(3-cholanamidopropyl)-dimethylammonio]-1-propane sulphate (CHAPS),
- b) centrifuge pipes and
- c) buoyant density solution with a density of 1.07 g/ml.

27. Kit according to Claim 26, characterized in that the lysis buffer consists of 109.5 g (0.32 M) sucrose, 1.221 g (10 mM) Tris-HCl, 10 ml (1%) Triton X-100, 1.016 g (5 mM) MgCl₂ to 1000 ml distilled water (pH 7.5),

28. Kit according to Claim 20 or 27, characterized in that the buoyant density solution consists of 10 ml (1.5 M) NaCl solution (87.6 g NaCl to 1000 ml), 49.2 ml Percoll with a density of 1.13 ± 0.005 g/ml and 40.8 ml distilled water (per 100 ml buoyant density solution).

29. Kits for the implementation of a method according to Claim 23 or 24, characterized in that it contains, in addition to the components of a kit according to Claims 26 to 28, components for the decomposition of bacteria and for DNA purification.

30. Kit according to Claim 29, characterized in that it contains Proteinase K.

31. Kit for the implementation of a method according to Claims 1 to 18, characterized in that it contains

- a) vessels and components for the implementation of a NAT, including the amplification primer, wherein the amplification primer contains sequences which are preserved in the case of the prokaryotes or eukaryotes in question to be detected,
- b) vessels and components for the implementation of one or more chain termination polymerization processes, including one or more (possibly labelled/differently labelled) sequencing primers (preferably with a length of 15 to 30 nucleotides), which hybridize with a region within the sequences to be amplified, which is preserved in the case of the prokaryotes or eukaryotes to be detected, and
- c) components for determination of the elongate length.

32. Kit according to Claim 31, characterized in that it contains, for the implementation of the chain termination polymerization, either 1, 2 or 3 of the four possible deoxyribonucleoside triphosphates (dNTPs) with one of these dNTPs being replaced by a chain-termination deoxyribonucleoside triphosphate.

33. Kit according to Claim 32, characterized in that the chain-termination deoxyribonucleoside triphosphate is a ddNTP, 3'-O-methyl-NTP or 3'-amino-NTP.

34. Kit according to Claims 31 to 33, characterized in that the components for determination of the elongate length include devices and means for implementation of an electrophoretic method, a mass-spectroscopic method or fluorescence-correlation spectroscopy.
35. Kit according to Claims 31 to 34, characterized in that it additionally contains the components of a kit according to Claims 25 to 29.
36. Kit according to Claims 31 to 35, characterized in that it is a kit for the detection of prokaryotes, which contains two highly-preserved sequences from the rRNA region as amplification primers.
37. Kit according to Claim 36, characterized in that the amplification primers are the primers according to SEQ ID NO 1 and 2 or the primers according to SEQ ID 3 and 4.
38. Kit according to Claims 36 and 37, characterized in that it contains at least one sequencing primer, which is hybridized with a preserved region of the rRNA region.
39. Kit according to Claim 38, characterized in that the sequencing primer(s) is/are chosen from the group consisting of sequencing primers according to SEQ ID NO 5, 6, 7, 8, 9, 10 and 11.
40. Kit according to Claim 39, characterized in that it contains a mixture of the sequencing primers according to SEQ ID NO 5 and 6, a mixture of the sequencing primers according to SEQ ID NO 7 to 9, and a mixture of the sequencing primers according to SEQ ID NO 10 and 11 in one single container or separate containers.

WO 01/07648

PCT/EP99/05234

-34-

41. Kit according to Claims 31 to 40, characterized in that it is a kit for the detection of sepsis inducers.

REPLACEMENT SHEET (RULE 26)

DECLARATION

Title: **METHOD FOR THE SPECIES-SPECIFIC DETECTION
OF ORGANISMS**

International Patent Application No. **PCT/EP99/05234**

International filing (priority) date: **July 22, 1999**

Entry into U.S. national stage as Serial No. **10/031,530**

U.S. National Stage entry date: **January 17, 2002**

U.S. Applicants / Inventors: **Krupp et al.**

Campbell & Flores Attorney Docket No. **P-UX 5156 (P 59364)**

I believe that I am an original inventor of the subject matter that is claimed and for which a patent is sought in the application identified above.

I hereby state that I have reviewed and understand the contents of the application identified above, including the specification and claims.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to myself to be material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56.

Under Sec. 1.56, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or (2) It refutes, or is inconsistent with, a position the applicant takes in: (a) Opposing an argument of unpatentability relied on by the U.S.

POWER OF ATTORNEY

Assignee **Artus Gesellschaft für molekularbiologische
Diagnostik und Entwicklung mbH**

is the owner of the entire right, title and interest of the following U.S. patent application identified below and any subsequently filed divisional, continuation, continuation-in-part or reissue applications claiming priority thereto.

Title: **METHOD FOR THE SPECIES-SPECIFIC DETECTION
OF ORGANISMS**

International Patent Application No. **PCT/EP99/05234**

International filing (priority) date: **July 22, 1999**


Entry into U.S. national stage as Serial No. **10/031,530**

U.S. National Stage entry date: **January 17, 2002**

U.S. Applicants / Inventors: **Krupp et al.**

Campbell & Flores Attorney Docket No. **P-UX 5156 (P 59364)**

The Assignee hereby appoints the following attorneys to prosecute these applications and to transact all related business in the United States Patent and Trademark Office:

 CATHRYN CAMPBELL, Registration No. 31,815;
DAVID A. GAY, Registration No. 39,200;
CALVIN A. FAN, Registration No. 38,444;
ANDREA L. GASHLER, Registration No. 41,029;
JAMES J. WONG, Registration No. 34,949;
DEBORAH L. CADENA, Registration No. 44,048;
MELANIE K. WEBSTER, Registration No. 45,201;
ASTRID R. SPAIN, Registration No. 47,956; and
KIMBERLY J. PRIOR, Registration No. 41,483.

Inventors: Krupp et al.
Serial No. 10/031,530
Int'l Filing Date: July 22, 1999
U.S. Nat'l Entry Date: January 17, 2002
Page 3

2.00 Full name of second original inventor: Peter Scheinert

Citizenship: Germany

Residence: Hamburg, Germany

Mailing Address: Övelgönner Str. 25, D-20257 Hamburg, Germany

3.00 Signature: X [Signature] Date: X 27/05/02

Full name of third original inventor: Rainer Söller

Citizenship: Germany

Residence: Bremen, Germany

Mailing Address: Lesmonastr. 18, D-28717 Bremen, Germany

Signature: X R. Söller Date: 27/05/02

4.00 Full name of fourth original inventor: Ulrich Spengler

Citizenship: Germany

Residence: Hamburg, Germany

Mailing Address: Bahrenfelder Kirchenweg 25, D-22761, Hamburg
Germany

Signature: X [Signature] Date: 27/05/02

Inventors: Krupp et al.
 Serial No. 10/031,530
 Int'l Filing Date: July 22, 1999
 U.S. Nat'l Entry Date: January 17, 2002
 Page 2

Patent and Trademark Office, or (b) Asserting an argument of patentability.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

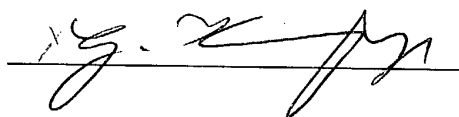
My citizenship, residence and mailing address are correctly stated below my name:

Full name of first original inventor: Guido Krupp

Citizenship: Germany

Residence: Gnutz, Germany

Mailing Address: Rosenstr. 3, D-24622, Gnutz, Germany

Signature:  Date: X 27/05/02

Inventors: Krupp et al.
Serial No. 10/031,530
Int'l Filing Date: July 22, 1999
U.S. Nat'l Entry Date: January 17, 2002
Page 2

Please direct all telephone calls to Cathryn Campbell at
(858) 535-9001 and address all correspondence to

~~CATHRYN CAMPBELL~~
~~CAMPBELL & FLORES LLP~~
~~4370 La Jolla Village Drive~~
~~7th Floor~~
~~San Diego, California 92122~~
~~USPTO CUSTOMER NO. 23601~~

The undersigned is empowered to sign this statement on behalf of
the assignee.

Date: X 27/05/02

Signature: Ulrich Spengler

Name: Dr. Ulrich Spengler

Title: C.E.O.

Artus Gesellschaft für
molekularbiologische
Diagnostik und Entwicklung mbH

Gerstäckerstrasse 9
D-20459 Hamburg, Germany

SEQUENCE LISTING

<110> Krupp Dr., Guido
Scheinert, Peter
Söller Dr., Rainer
Spengler Dr., Ulrich
Artus Gesellschaft für molekularbiologische
Diagnostik und Entwicklung mbH

<120> Method for the species-specific detection of organism

<130> P050133

<140>

<141>

<160> 11

<170> PatentIn Vers. 2.0

<210> 1

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
16S-5'terminal primer

<400> 1

agagtttgat cmtggctcag

20

<210> 2

<211> 22

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
16S-3'terminal primer

<400> 2

tacggytacc ttgttacgac tt

22

<210> 3
<211> 18
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence: 16S-proximal
primer

<400> 3
aagtcgtaac aaggtarc

18

<210> 4
<211> 15
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence: 23S-proximal
primer

<400> 4
ggttbcccaca ttcrg

15

<210> 5
<211> 17
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
Sequencing primer 109r1

<400> 5
acgygttack cacccgt

17

<210> 6
<211> 17
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
Sequencing primer 109r2

<400> 6

akrcattact caccgt

17

<210> 7

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:

Sequencing primer 685r1

<400> 7

tctacgratt tcaccyctac

20

<210> 8

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:

Sequencing primer 685r2

<400> 8

tctacgcatt tcacygctac

20

<210> 9

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Description of the artificial sequence:

Sequencing primer 685r3

<400> 9

tctrcgcatt ycaccgctac

20

<210> 10
<211> 18
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Sequencing primer 1475rs

<400> 10
cccaccttcg acggctag

18

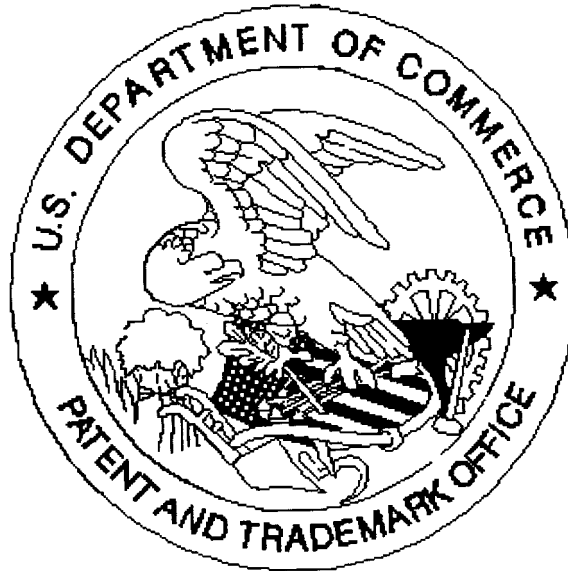
<210> 11
<211> 18
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Sequencing primer 1475rh

<400> 11
cataccgtgg taaacgcc

18

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not
present
for scanning. (Document title)

*page 35, to page 38, page specification
are sequence listing*

☐ *Scanned copy is best available.*